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In re application of:	Confirmation No.: 4611
CAI *et al.*	Art Unit: 1625
Appl. No.: 10/733,229	Examiner: Aulakh, C.
Filed: December 12, 2003	Atty. Docket: 1735.0810001/RWE/BSA
For: **Substituted 1-Benzoyl-3-Cyano-pyrrolo[1,2-A]quinolines and Analogs as Activators of Caspases and Inducers of Apoptosis**	

Declaration Under 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Sui Xiong Cai, declare:

1. I am the inventor of the subject matter claimed in U.S. application no. 10/733,229 (the '229 application).

2. In an Office Action dated July 15, 2005 claims 1-3, 5-11, 13-22, 27-42, 45 and 46 of the '229 application were rejected for lack of enablement on the ground that the specification does not enable treating or ameliorating any cancer or disorder responsive to the induction of apoptosis.

3. The experiments described below provide further proof that caspase activators of the present invention induce apoptosis in a wide variety of cancer cells.

4. In this set of experiments, additional examples are provided to demonstrate that the caspase activators of the present invention induce apoptosis in human breast cancer cell lines T-47D and ZR-75-1, human colon cancer cell lines DLD-1 and HT29, and human lung cancer cell line H1299.

5. Human breast cancer cell lines T-47D and ZR-75-1, human colon cancer cell lines DLD-1 and HT29, and human lung cancer cell line H1299 were grown according to media component mixtures designated by American Type Culture

Collection + 10 % FCS (Invitrogen Corporation), in a 5 % CO_2-95 % humidity incubator at 37 °C. These cancer cells were maintained at a cell density between 30 and 80 % confluency and for HL-60 at a cell density of 0.1 to 0.6 x 10^6 cells/mL. Cells were harvested at 600xg and resuspended at 0.65 x 10^6 cells/mL into appropriate media + 10 % FCS. An aliquot of 45 µL of cells was added to a well of a 96-well microtiter plate containing 5 µL of a 10 % DMSO in RPMI-1640 media solution containing 1.6 to 100 µM of 1-benzoyl-3-cyano-pyrrolo[1,2-*a*]quinoline or other test compound (0.16 to 10 µM final). An aliquot of 45 µL of cells was added to a well of a 96-well microtiter plate containing 5 µL of a 10 % DMSO in RPMI-1640 media solution without test compound as the control sample. The samples were mixed by agitation and then incubated at 37 °C for 24 h or 48 h in a 5 % CO_2-95 % humidity incubator. After incubation, the samples were removed from the incubator and 50 µL of a solution containing 20 µM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 fluorogenic substrate (SEQ ID NO:1), 20 % sucrose (Sigma), 20 mM DTT (Sigma), 200 mM NaCl (Sigma), 40 mM Na PIPES buffer pH 7.2 (Sigma), and 500 µg/mL lysolecithin (Calbiochem) was added. The samples were mixed by agitation and incubated at room temperature. Using a fluorescent plate reader (Model 1420 Wallac Instruments), an initial reading (T = 0) was made approximately 1-2 minutes after addition of the substrate solution, employing excitation at 485 nm and emission at 530 nm, to determine the background fluorescence of the control sample. After approximately 3 h of incubation, the samples were read for fluorescence as above (T = 3 h).

6. Calculation:

The Relative Fluorescence Unit values (RFU) were used to calculate the sample readings as follows:

$$RFU_{(T=3h)} - \text{Control } RFU_{(T=0)} = \text{Net } RFU_{(T=3h)}$$

7. The activity of caspase cascade activation was determined by the ratio of the net RFU value for 1-benzoyl-3-cyano-pyrrolo[1,2-*a*]quinoline to that of control samples. The EC_{50} (nM) was determined by a sigmoidal dose-response calculation (Prism 2.0, GraphPad Software Inc.). The caspase activity (Ratio) and potency (EC_{50}) are summarized in Table I:

Table I. Caspase Activity and Potency

Example #	T-47D EC_{50} (nM)	ZR-75-1 EC_{50} (nM)	DLD EC_{50} (nM)	H1299 EC_{50} (nM)	HT29 EC_{50} (nM)
Example A	243	243	2853	1902	1575
1	157	133	920	ND	ND
4	463	624	242	721	286
5	ND	159	197	ND	ND
7	351	185	693	ND	ND
8	587	2582	>10000	>10000	>10000
9	150	150	1159	849	>10000
11	148	147	379	ND	ND
12	1163	493	702	ND	ND
14	550	310	1280	ND	ND
15	613	529	2384	ND	ND
18	316	534	1954	ND	ND
19	31	78	312	301	555
21	41	46	122	ND	ND
22	277	122	350	239	384
25	474	3552	1723	ND	ND
29	134	149	1420	ND	ND
34	41	44	238	ND	ND
35	303	279	1323	283	>10000
36	216	87	170	ND	ND
37	38	121	346	>10000	62
38	284	312	1273	371	>10000
43	212	193	469	671	529
46	1027	194	633	ND	ND
48	922	ND	297	458	5217
59	118	105	145	ND	ND
77	158	ND	557	3028	ND

Example #	T-47D EC_{50} (nM)	ZR-75-1 EC_{50} (nM)	DLD EC_{50} (nM)	H1299 EC_{50} (nM)	HT29 EC_{50} (nM)
78	124	ND	344	1474	1600
79	297	ND	>10000	1399	ND

ND, not determined.

8. Thus, the data show that 1-benzoyl-3-cyano-pyrrolo[1,2-*a*]quinoline (Example A) and other analogs are potent caspase cascade activators and antineoplastic compounds in this assay.

9. In another set of experiments, additional examples are provided to demonstrate that the caspase activators of the present invention induce apoptosis in human breast cancer cell lines T-47D, MX-1 and SKBr-3, human colon cancer cell lines DLD-1 and HT29, human lung cancer cell line H1299, human prostate cancer cell line LNCaP, and murine pre-B cell lymphoma cell line P388.

10. Human breast cancer cell lines T-47D, MX-1 and SKBr-3, human colon cancer cell lines DLD-1 and HT29, human lung cancer cell line H1299, human prostate cancer cell line LNCaP, and murine pre-B cell lymphoma cell line P388 were grown and harvested as described above. An aliquot of 90 μL of cells (2. 2 x 10^4 cells/mL) was added to a well of a 96-well microtiter plate containing 10 μL of a 10 % DMSO in RPMI-1640 media solution containing 1 nM to 100 μM of 1-benzoyl-3-cyano-pyrrolo[1,2-*a*]quinoline (0.1 nM to 10 μM final). An aliquot of 90 μL of cells was added to a well of a 96-well microtiter plate containing 10 μL of a 10 % DMSO in RPMI-1640 media solution without compound as the control sample for maximal cell proliferation (A_{Max}). The samples were mixed by agitation and then incubated at 37 °C for 48 h in a 5 % CO_2-95 % humidity incubator. After incubation, the samples were removed from the incubator and 20 μL of CellTiter 96 AQUEOUS One Solution Cell Proliferation™ reagent (Promega) was added. The samples were mixed by agitation and incubated at 37 °C for 2-4 h in a 5 % CO_2-95 % humidity incubator. Using an absorbance plate reader (Model 1420 Wallac Instruments), an initial reading (T = 0) was made approximately 1-2 min after addition of the solution, employing absorbance at 490 nm. This determines the possible background

absorbance of the test compounds. No absorbance for 1-benzoyl-3-cyano-pyrrolo[1,2- *a*]quinoline was found at 490 nm. After the 2-4 h incubation, the samples were read for absorbance as above (A_{Test}).

11. Baseline for GI_{50} (dose for 50 % inhibition of cell proliferation) of initial cell numbers were determined by adding an aliquot of 90 μL of cells or 90 μL of media, respectively, to wells of a 96-well microtiter plate containing 10 μL of a 10 % DMSO in RPMI-1640 media solution. The samples were mixed by agitation and then incubated at 37 °C for 0.5 h in a 5 % CO_2-95 % humidity incubator. After incubation, the samples were removed from the incubator and 20 μL of CellTiter 96 AQUEOUS One Solution Cell Proliferation™ reagent (Promega) was added. The samples were mixed by agitation and incubated at 37 °C for 2-4 h in a 5 % CO_2-95 % humidity incubator. Absorbance was read as above, (A_{Start}) defining absorbance for initial cell number used as baseline in GI_{50} determinations.

12. Calculation:

GI_{50} (dose for 50 % inhibition of cell proliferation) is the concentration where $[(A_{Test} - A_{Start}) / (A_{Max} - A_{Start})] = 0.5$.

13. The GI_{50} (nM) are summarized in Table II:

Table II. GI_{50} in Cancer Cells

Example #	T-47D EC_{50} (nM)	DLD EC_{50} (nM)	LnCap EC_{50} (nM)	MX-1 EC_{50} (nM)	SKBR3 EC_{50} (nM)	P388 EC_{50} (nM)	H1299 EC_{50} (nM)	HT29 EC_{50} (nM)
A	30	3500	ND	10000	32	2000	2611	3271
1	36	46	17	96	42	35	119	512
4	138	1347	4444	438	93	1621	540	483
5	7929	250	690	48	368	309	ND	ND
7	1097	592	2007	884	114	248	ND	ND
8	100	5424	>10000	114	ND	>10000	10922	6893
9	392	769	1600	91	ND	6347	1000	1000
11	295	2485	362	965	ND	535	ND	ND

Example #	T-47D EC_{50} (nM)	DLD EC_{50} (nM)	LnCap EC_{50} (nM)	MX-1 EC_{50} (nM)	SKBR3 EC_{50} (nM)	P388 EC_{50} (nM)	H1299 EC_{50} (nM)	HT29 EC_{50} (nM)
12	350	5367	1216	913	296	316	ND	ND
14	941	3362	>10000	1241	ND	7717	ND	ND
19	11	716	>10000	280	ND	>10000	77660	800
21	53	84	162	89	ND	652	ND	ND
22	274	633	76	581	ND	207	ND	ND
25	928	590	ND	300	ND	ND	581	ND
29	11281	450	ND	211	ND	ND	>10000	ND
34	38	151	ND	430	ND	ND	ND	ND
35	408	3401	24941	695	ND	954	ND	ND
36	607	501	654	1300	ND	338	ND	ND
37	223	182	>10000	127	ND	>10000	>10000	500
38	445	1800	1685	928	ND	6592	944	3000
43	626	444	1327	1629	ND	479	ND	ND
46	943	896	1624	5550	ND	712	ND	ND
48	200	ND	ND	591	ND	ND	208	5000
59	946	532	ND	911	ND	ND	348	ND
77	7496	1055	ND	662	ND	ND	4497	ND
79	1768	7688	ND	7932	ND	ND	ND	ND

ND, not determined.

14. Thus, These data show that 1-benzoyl-3-cyano-pyrrolo[1,2-*a*]quinoline (Example A) and analogs are antineoplastic compounds that inhibit cell proliferation.

15. In sum, these experiments demonstrate that 1-benzoyl-3-cyano-pyrrolo[1,2-*a*]quinoline and analogs are potent caspase cascade activators and antineoplastic compounds active against multiple cancer cell lines.

16. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both,

under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 10/03/05

Sui Xiong Cai, Ph.D.